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(54) Title: MANIPULATION OF OXIDATIVE PHOSPHORYLATION FOR HYPERSENSITIZING TUMOR CELLS TO GLYCOLYTIC INHIBITORS

(57) Abstract: A class of glycolytic inhibitors is useful in the treatment of solid tumors by attacking anaerobic cells at the center on the tumor. 2-deoxyglucose, oxamate and various analogs thereof are identified as having a natural selective toxicity toward anaerobic cells, and will significantly increase the efficacy of standard cancer chemotherapeutic and radiation regimens as well as new protocols emerging with anti-angiogenic agents.

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**MANIPULATION OF OXIDATIVE PHOSPHORYLATION FOR
HYPERSENSITIZING TUMOR CELLS TO GLYCOLYTIC INHIBITORS**

5 **FIELD AND BACKGROUND OF THE INVENTION**

The present invention relates, in general, to compositions and methods aimed at effectively treating anaerobic tumor cells with inhibitors of glycolysis. It also extends to novel and useful methods and compositions for
10 treating aerobic tumor cells with inhibitors of oxidative phosphorylation in combination with glycolytic inhibitors. We discovered that inhibiting oxidative phosphorylation to convert aerobic tumor cells to anaerobic cells, hypersensitizes them to glycolytic inhibitors.

15 U.S. patents that are relevant to the compositions and methods of the present invention are: U.S. Patents 4,840,939 and 4,684,627, both for a treatment of cancer with phlorizin and its derivatives; U.S. Patent 4,683,222 for N-glycosylated carboxamide derivatives and their use for influencing the
20 body's inherent defenses; and U.S. Patent 4,420,489 for sugars with sulfur replacing the ring oxygen atom as antiradiation agents. U.S. Patent 4,840,939 is also relevant for its discussion of the work of Warburg in 1931, concerning the way cancer cells metabolize glucose.

25 Cancer cells at the inner core of a tumor are poorly oxygenated and consequently rely on anaerobic metabolism for

survival. In this condition tumor cells divide more slowly than outer growing aerobic cells and consequently are more resistant to standard chemotherapeutic agents which target rapidly dividing cells. Thus, cells growing anaerobically in these instances exhibit a form of multidrug resistance (MDR) which contributes to chemotherapy failures in the treatment of solid tumors. Anaerobiosis, however, also provides a natural window of selectivity for agents that interfere with glycolysis.

10 This realization forms the basis for the present invention. According to the present invention, new opportunities are provided for increasing the efficacy of chemotherapeutic protocols. With data and knowledge accumulated by us in our previous work on mitochondrial agents, and with additional work performed by us now, various hypotheses have been formulated and verified to prove the efficacy of the present invention with regard to its compositions and its methods.

20 See, for example, a series of papers coauthored by one of the present inventors: Lampidis, TJ, Bernal, SD, Summerhayes, IC, and Chen, LB. "Rhodamine 123 is selectively toxic and preferentially retained in carcinoma cells in vitro." NY Acad. Sci. 397:299-302, 1982; Summerhayes, IC, Lampidis, TJ, Bernal, SD, Shepherd, EL, and Chen, LB. "Unusual retention of Rhodamine 123 by mitochondria in muscle and carcinoma cells." Proc. Natl. Acad. Sci. USA 79:5292-5296, 1982; Bernal, SB, Lampidis, TJ, Summerhayes, IC, and Chen, LB. "Rhodamine 123 selectively reduces clonogenic ability of carcinoma cells in vitro." Science. 218:1117-1119, 1982; Lampidis, TJ, Bernal, SD, Summerhayes, IC, and Chen, LB. "Selective toxicity of Rhodamine 123 in carcinoma cells in vitro." Cancer Res. 43:716-720, 1983; and Bernal, SB, Lampidis, TJ, McIsaac, B,

and Chen, LB. "Anticarcinoma activity in vivo of Rhodamine 123, a mitochondrial-specific dye." Science. 222:169-172, 1983; which showed that Rhodamine 123 (Rho 123) which localizes in mitochondria of living cells, and uncouples ATP synthesis from electron transport, preferentially accumulates in, and kills, a variety of tumor cells as compared to a number of normal cells. We reasoned for the present invention that tumor cells treated with this drug would have to rely solely on glycolysis for ATP production and thus become hypersensitized to inhibitors of glycolysis, like 2-dg (2-dg). In contrast, mitochondrial function in normal cells remained unaffected when treated with Rho 123 and therefore these cells were not hypersensitive to 2-dg. In fact, we found that co-treating human breast carcinoma cells, MCF-7, with Rho 123 and 2-dg, at a dose of Rho 123 that alone inhibited 50% of colony forming units, and at a dose of 2-dg which produced no toxicity, 100% of the colony forming units was inhibited.

This concept was carried over to *in vivo* studies in which it was found that animals with implanted tumors that were treated in combination with 2-dg and Rho 123 were cured whereas when treated with either drug alone, only partial or no responses were obtained. This latter result provides evidence that manipulation of Oxphos and glycolysis simultaneously can cure tumors in animals. Furthermore, this *in vivo* data also demonstrates that 2-deoxyglucose can be administered safely to animals, at doses which are effective for anti-tumor activity in combination with an Oxphos inhibitor. In this regard, several reports have shown that low levels of 2-dg can be safely administered to animals for various reasons including hypersensitization of tumors to irradiation.

SUMMARY OF THE INVENTION

The present invention is based on the discovery that glycolytic inhibitors and analogs thereof, are selectively toxic to tumor cells which are metabolizing anaerobically. Thus, in conjunction with standard chemotherapy and/or radiation which is focused on aerobic, fast-growing cells, which we will here refer to collectively and individually as "aerobic treatment", use of these inhibitors will add to the efficacy of cancer treatment by selectively killing the anaerobically slow-growing tumor cells found at the inner core of solid tumors which are usually the most resistant and consequently the most difficult to eradicate using aerobic treatments.

As an aid for explaining and for demonstrating the usefulness of the present invention, our discovery can be summarized by the equation $(A)=(B)=(C)$ where:

(A) = tumor cells treated at a dose of rhodamine 123 which specifically uncouples ATP synthesis from electron transport;

(B) = ρ^0 cells which are cells that contain no mitochondrial DNA and therefore cannot undergo oxidative phosphorylation; and

(C) = tumor cells which are growing anaerobically.

In all three classes, the cells can only produce ATP via the Embden-Myerhoff pathway (glycolysis) and thus are naturally hypersensitive to all inhibitors of glycolysis exemplified by 2-deoxyglucose, oxamate and novel compounds of the present invention.

Although Warburg originally proposed that tumor cells depend less on mitochondrial function for ATP production and more on glycolysis than normal cells, there has been no

definitive data to date to confirm this hypothesis. There is data however which indicates that cells, tumor or normal, which are compromised aerobically and switch to anaerobic metabolism increase their uptake and utilization of glucose.

5 The increased uptake has been attributed to the increased appearance of glucose receptors on the plasma membrane. The fact that cells growing at the inner core of tumors rely more on glycolysis than cells on the outer edge most likely accounts for the successful use of the radioactive analog of

10 glucose, 2-deoxyglucose (2-dg), as a diagnostic tool for localizing tumors. This has been the main usage of 2-dg for cancer. Moreover, it has been suggested that 2-dg is taken up more by anaerobic cells due to enhanced expression of glucose receptors. Thus, since a cell that is functioning normally

15 relies mainly on oxidative phosphorylation for its supply of ATP when this mechanism is compromised or absent, glycolysis (the only other way of producing ATP) automatically becomes enhanced. This is precisely why inner core tumor cells should naturally become hypersensitive to agents which block

20 glycolysis, i.e. 2-dg and others that block the glycolytic pathway at different steps, such as oxamate and iodoacetate.

Oxamate is another agent which blocks glycolysis and is more specific than 2-dg for anaerobically metabolizing cells. Oxamate has been shown to inhibit lactic dehydrogenase, the

25 enzyme which breaks down pyruvate to lactic acid. Thus, the fact that oxamate blocks glycolysis at a different step than 2-dg, and is clearly active in our in vitro cell systems in selectively killing anaerobic cells (models A and B), lends further proof that our discovery is correct and works

30 according to the principles we set forth in this application. The enzyme reaction which converts pyruvate to lactate occurs only when the cell's ability to process oxygen becomes limited. Thus, aerobically metabolizing cells do not use this

enzyme and inhibitors of this reaction will inherently be more selective than inhibitors of glycolysis such as 2-deoxyglucose since these latter agents inhibit reactions that occur in aerobic as well as anaerobic cells.

5 Iodoacetate, on the other hand, is known to inhibit glycolysis at another step in the pathway, namely at glyceraldehyde 3-phosphate dehydrogenase, which normally yields 1,3 biphosphoglycerate from glyceraldehyde 3-phosphate and requires NAD⁺ to proceed.

10 The inventors have investigated this overall concept by utilizing an osteosarcoma cell line that has been selected for loss of mtDNA, and thus cannot produce ATP by oxidative phosphorylation (cells (B) above). The inventors have also utilized this concept as a model to determine whether any test
15 drug utilizes functional mitochondria as a cytotoxic target. The model indicates that a drug which interferes with ATP production in mitochondria of whole cells, but not necessarily in isolated mitochondrial preparations, will hypersensitize a cell to glycolytic inhibitors, i.e. 2-dg, oxamate and
20 iodoacetate. Data has been developed by the inventors which clearly shows that in the cases listed as (A) and (B) above, both are hypersensitive to 2-deoxyglucose oxamate and iodoacetate which forms the basis for the present invention.

The data which supports the invention that anaerobic
25 cells within a tumor are selectively sensitive to glycolytic inhibitors, indicates that this is a universal phenomenon and that most or all glycolytic inhibitors will, in general, be similarly effective.

The various features of novelty which characterize the
30 invention are pointed out with particularity in the claims annexed to and forming a part of this disclosure. For a better understanding of the invention, its operating advantages and specific objects attained by its uses,

reference is made to the accompanying descriptive matter in which preferred embodiments of the invention are illustrated.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention identifies classes of novel
5 compositions and novel methods of using them, as well as novel methods of using classes of known compositions to kill anaerobic tumor cells and to enhance the effectiveness of aerobic treatments for killing aerobic tumor cells.

The inventors have identified six categories of
10 glycolytic inhibitors that can be used according to the present invention.

1. Analogs that increase tumor targeting and uptake:

(a) Lipophilic analogs of 2-deoxy-D-glucose.

In order to increase the uptake of 2-deoxyglucose into
15 the inner core of slow growing anaerobic cells, increases in lipophilicity aids in penetration and therefore higher levels reaching these inner cells. In part, such analogs might also work as lipophilic prodrugs of 2-deoxy-D-glucose.

Examples: Lipophilic analogs include derivatives of
20 hydroxyl groups like esters, ethers, phosphoesters, etc. Others include the removal of the hydroxyl group and replacement with halogens like fluorine or iodine, or with thiol or thioalkyl groups.

(b) Liposome formulated 2-deoxy-glucose and its analogs
25 (also inhibitors of glycolysis such as oxamate and iodoacetic acid). Due to the ability to change the lipophilic nature of the outer surface of liposomes and entrap drugs for better delivery to different tissue sites, liposomes have previously

been shown to increase tumor uptake of a variety of different drugs and molecules in experimental tumor models.

(c) Enzymatically cleavable derivatives of 2-deoxyglucose.

5 Examples of this class of compounds include Glucoronides with glycosides at the C-1 position. The rationale of the invention is that the level of the enzyme glucoronidase has been shown to be higher in tumors than in normal tissues. Thus, by synthesizing 2-deoxyglucose in the form of a
10 glucoronide its concentration at the tumor site will be naturally enhanced by the increased levels of glucoronidases which will cleave it and deliver active 2-deoxyglucose specifically to the tumor.

2. Analogs of D-hexopyranoses:

15 For this disclosure the word "hexopyranoses" is used to cover all configurational isomers of monosaccharides modified at C-6 by removal (replacement) of 6-OH or by blocking it.

Such analogs will not be transformed by hexokinase or glucokinase to glucose- 6-phosphate and can potentially block
20 both enzymes.

Examples: 6-deoxy-D-glucose, 6-fluoro-D-glucose, and 6-O-methyl-D-glucose.

3. Analogs blocking transformation from glucose 6-phosphate to fructose 6-phosphate:

25 (a) Such transformation requires the presence of an hydroxyl group at C-2. Therefore, analogs without hydroxyl or having the hydroxyl properly blocked easily undergo the first step of 6-O-phosphorylation by hexokinase or glucokinase but will not undergo isomerization by phosphoglucose isomerase.
30 In fact such compounds can efficiently block the enzyme.

Examples: 2-deoxy-D-glucose itself and its analogs. Other useful analogs are 2-deoxy-2-halo-D-glucose, for example 2-fluoro- or 2-iodo-D-glucose.

(b) Another way to stop isomerization is modification at
5 C-1. Replacement of hydroxyl by fluorine (glucosyl fluoride) or simple deoxygenation to 1-deoxy-D-glucose.

4. Analogs blocking the aldolase A cleavage, thus blocking formation of trioses from fructose 1,6-bisphosphate:

Such a process requires the presence of hydroxyl groups
10 at C-3 and C-4. Thus, for example, 3-deoxy or 3-fluoro-D-glucose or 4-deoxy or 4-fluoro-D-glucose can be transformed to 4-fluoro-D-fructose 1,6-bisphosphate, which will not be cleaved by aldolase A but, will block it.

5. Blocking any of the transformations of glyceraldehyde:

15 Alterations are made at any step where phosphorylation is involved. Here either 2-fluoro or 3-fluoro-glyceraldehydes or glycerates can be used. If the phosphoester is important for blocking the enzyme, then, for example, 3-fluoro-2-phosphoglycerate is used to block transformation to
20 phosphoenolpyruvate

Also, phosphothioesters or other phosphor modified analogs can block the transformations of glyceraldehyde.

6. The pyruvate structure:

Keeping in mind that oxamate is a good inhibitor of
25 lactate dehydrogenase, compositions or structures of the present invention which are efficient blockers of lactate dehydrogenase are included. An example is 2-fluoro-propionic acid or its salts; 2,2-difluoro-propionic acid.

Other examples are a pyruvate modified at C-3 such as 3-

halo-pyruvate; 3-halopropionic acid, 2-thiomethylacetic acid.

In addition to the proposed use of inhibitors of glycolysis as supplements to current chemotherapeutic and radiation treatment, the recent emergence of anti-angiogenic factors aimed at cutting off the blood supply to tumors and thus creating a more anaerobic environment, indicates that the present invention will likely have applicability to this newly developing cancer therapy as well.

General Synthesis of Novel Chemicals:

10 The following section discloses the synthesis of analogs of glycolytic intermediates and determines their toxic potencies in ρ^0 cells and in tumor cells pretreated with Oxphos inhibitors.

15 According to the invention, a series of compounds has been rationally designed and prepared, that can block various steps of the glycolytic pathway, and which thus have an effect on cell models A, B, and C.

20 It is commonly believed that the three irreversible reactions of glycolysis catalyzed by hexokinase, phosphofructokinase, and pyruvate kinase are the controlling elements of this pathway. Thus, it would appear that the design and synthesis of inhibitors should focus on these steps. However, due to the complexity of interactions between glycolysis and other pathways it is likely that restricting the focus to the controlling elements is too naive. Thus, we will consider all reactions in the glycolytic pathway as potential targets and design compounds accordingly.

25 Specifically, we have designed and synthesized the following, noting that the number in parenthesis after each analog corresponds to the structure illustrated in the following pages

Hexokinase blockers.

6-Fluoro-D-glucose (1) and 6-thio-D-glucose (2) are synthesized. Removal of the hydroxyl group from the C-6 carbon in glucose and replacement with fluorine (6-fluoro-D-glucose) and replacement with a thiol group respectively. 6-fluoro-D-glucose should prevent formation of glucose 6-phosphate and inhibit hexokinase. 6-Thio-D-glucose (2) behaves similarly to 6-fluoro-D-glucose (1) or may be transformed into a 6-thiophosphate derivative. In either case, they effectively block glycolysis.

Analogs blocking transformation from glucose 6-phosphate to fructose 6-phosphate.

Transformation of glucose 6-phosphate to fructose 6-phosphate requires a hydroxyl group at C-2 in the glucose molecule. Therefore, analogs lacking a hydroxyl in this position, or having a properly blocked hydroxyl might easily undergo 6-O-phosphorylation by hexokinase but will not be able to undergo isomerization by phosphoglucose isomerase to fructose 6-phosphate. In fact, 2-dg is an example of such a glucose analog that efficiently stops glycolysis at the isomerization step. Other potentially useful C-2-modified analogs that can be prepared are 2-bromo-D-glucose, 2-fluoro-D-glucose, and 2-iodo-D-glucose (4-5).

In addition, it should be possible to stop isomerization via modification at C-1 or C-5. In the case of C-1, the hydroxyl at C-1 is replaced with fluorine glucosyl fluoride (7) or with hydrogen to produce 1-deoxy-D-glucose. In the case of C-5, sulfur replaces oxygen in the hexopyranose ring of glucose to obtain 5-thio-D-glucose (8).

Analogs blocking aldolase-catalyzed cleavage.

In the next step of glycolysis, fructose-6-phosphate is transformed to fructose 1,6-biphosphate. Subsequently,

fructose 1,6-biphosphate undergoes aldolase catalyzed cleavage to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The cleavage reaction requires that fructose 1,6-biphosphate contains hydroxyl groups at positions C-3 and C-4.

5 To exploit this requirement, we synthesize two glucose analogs modified with fluorine atoms at these positions, i.e. C-3 (3-fluoro-D-glucose) (9) and C-4 (4-fluoro-D-glucose) (10). These analogs are expected to undergo uninterrupted glycolysis to 3-fluor-fructose 1,6 biphosphate and 4-fluoro-, fructose

10 1,6-bisphosphate; however, neither of them should be able to undergo aldolase-catalyzed cleavage to trioses. Increased accumulation of such compounds should stop glycolysis at this step.

Analog blocking glyceraldehyde 3-phosphate

15 *transformation to pyruvate.*

Steps that occur later in the glycolytic pathway i.e. from glyceraldehyde 3-phosphate to pyruvate, are also targets for inhibitors of glycolysis. The transformation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate

20 catalyzed by glyceraldehyde 3-phosphate dehydrogenase can be blocked by 1,1-difluoro-3-phosphate-glycerol (11) and 1,1-difluoro-glycerate (12) is synthesized. Furthermore, analogs modified at C-2 are prepared with the rationale that substitution at this position will affect the action of either

25 one or all of the following enzymes, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, and phosphoglyceromutase. Examples of these potential glycolytic blockers are 2-fluoro-, 2-iodo-, 2-thio, or 2-methoxy-glyceraldehydes (13-16) or glycerates (17-20).

30 Considering the next step in the glycolytic pathway C-3-substituted trioses will have inhibitory properties by virtue of being able to block the catalyzed step. The analogs are 3-

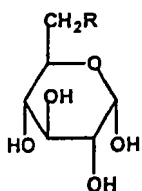
fluoro-, 3,3-difluoro-, enolase 3-iodo-, 3-carboxylo- and 3-thioglycerates (21-24).

Increasing the lipophilicity of 2-dg

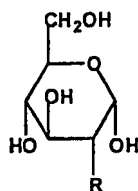
Lipophilic analogs of 2-dg act as prodrugs.
5 Specifically, mono and diesters of 2-dg are designed for increased uptake in tumor cells. Such lipophilic esters enter the cell via passive diffusion instead of relying on glucose transporters and then are hydrolysed by esterases, leading to the release of 2-dg. Valerate, myristate, and palmitate are
10 examples of lipophilic derivatives of 2-dg (26-37). Specifically useful are all four possible monoesters of 2-dg for each acid.

The structures of the inhibitors of the present invention are:

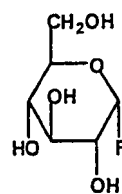
14



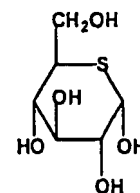
1 - R = F (6-Fluoro-D-glucose)
2 - R = SH (6-Thio-D-glucose)



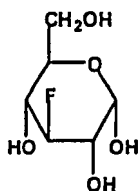
3 - R = H - 2-Deoxy-D-glucose (2-dg)
4 - R = Br - 2-Bromo-D-glucose
5 - R = F - 2-Fluoro-D-glucose
6 - R = I - 2-Iodo-D-glucose



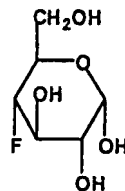
7 - Glucosyl fluoride



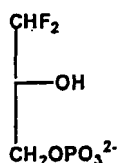
8 - 5-Thio-D-glucose



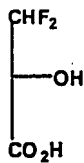
9 - 3-Fluoro-D-glucose



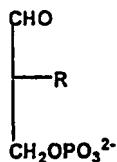
10 - 4-Fluoro-D-glucose



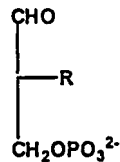
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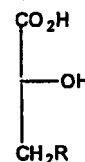
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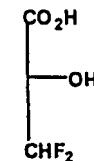
13 - R = I
14 - R = F
15 - R = SH
16 - R = OMe



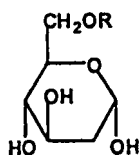
17 - R = I
18 - R = F
19 - R = SH
20 - R = OMe



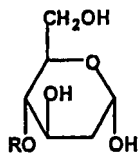
21 - R = I
22 - R = F
23 - R = SH
24 - R = CO₂H



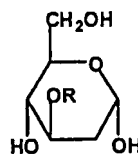
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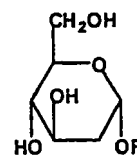
6-O-Esters of 2-dg
26 - R = CO(CH₂)₃CH₃
27 - R = CO(CH₂)₁₂CH₃
28 - R = CO(CH₂)₁₄CH₃



4-O-Esters of 2-dg
29 - R = CO(CH₂)₃CH₃
30 - R = CO(CH₂)₁₂CH₃
31 - R = CO(CH₂)₁₄CH₃



3-O-Esters of 2-dg
32 - R = CO(CH₂)₃CH₃
33 - R = CO(CH₂)₁₂CH₃
34 - R = CO(CH₂)₁₄CH₃



1-O-Esters of 2-dg
35 - R = CO(CH₂)₃CH₃
36 - R = CO(CH₂)₁₂CH₃
37 - R = CO(CH₂)₁₄CH₃

Diesters, however, are also useful. The pharmacokinetics of these mono and diester analogs of 2-dg, are different than that of the parent compound.

Test Results on Type (B) ρ^0 Cells.

When an osteosarcoma cell line (143B; Wild type in Table 1 below) was exposed to prolonged periods to ethidium bromide a mutant completely devoid of mitochondrial DNA ρ^0 was isolated. As a consequence this mutant cell line, referred to as either ρ^0 or cell line 206, cannot form intact respiratory chain complexes and therefore does not undergo electron transport and or oxidative phosphorylation. These processes are necessary for transmembrane proton pumping which creates an electronegative membrane potential ($\Delta\Psi_{mt}$) in the matrix side of the inner mitochondrial membrane. Since Rho 123 is a fluorescent dye, which due to its chemical positive charge accumulates in mitochondria, it is commonly used as an indicator of $\Delta\Psi_{mt}$. Thus, our results which show that the majority of ρ^0 cells stained dimly, while wt cells stained brightly after a 10 min. exposure to Rho 123, indicate that ρ^0 cells do indeed have an $\Delta\Psi_{mt}$, albeit reduced when compared to that in wt cells.

A mechanism by which ρ^0 cells are able to generate a $\Delta\Psi_{mt}$, has recently been proposed to occur via reversal of ATP transport into mitochondria through the adenine nucleotide transporter (ANT)). Thus, by exchanging ADP for ATP, the internal environment of mitochondria becomes more electronegative. This process, however, does not appear to restore the $\Delta\Psi_{mt}$, to its normal value.

Flow cytometric analysis was performed in order to quantitate the differences observed microscopically between wild type 143B (wt) and ρ^0 cells (designated as cell line 206) in their accumulation of Rho 123. A four-fold decrease in Rho 123 retention in ρ^0 cells as compared to the parental wt has been observed. This data agrees with results from other investigators working with these same cells (Bochet and

Godinet, and Barrientos and Moraes), in which they estimate that $\Delta\Psi_{mt}$ of the ρ^0 cells to be 3-4 times lower than wt.

To ensure that our accumulation results were not complicated by P-gp-mediated MDR, since Rho 123 is a known substrate for this transporter, both cell types were examined for this gene by RT-PCR and were found not to express it. An interesting observation which may have significance for clinical application of Rho 123 and verapamil for detecting P-gp in freshly excised tumors or tumor samples from cancer patients is that in these non-P-gp cell lines, verapamil increases the retention of Rho 123.

The ρ^0 cell model as a tool to determine whether drugs use functional mitochondria as a cytotoxic target.

Drugs which depend on functional mitochondria for localization, accumulation or for free radical formation, should be less potent in ρ^0 than in equivalent wt cells. Thus, it is not surprising to find that in growth inhibition studies, as shown in **Table 1**, ρ^0 cells are 50 and 6 times more resistant to Rho 123 and saffranin O (another mitochondrial cationic dye) than wt cells, respectively.

Table 1
50% Growth Inhibition Dose ($\mu\text{g/ml}$)
(CELLS)

	Drugs	Wild type	ρ^0	Ratio
5				
	Rhodamine 123	1.0	50	50
	Saffranin O	0.35	2	6
	Doxorubicin	0.01	0.01	1
	Vinblastine	0.00075	0.00075	1
10	Paclitaxel	0.05	0.05	1
	2-deoxy-glucose	1000	50	0.05

The differences between wt and ρ^0 cells in their sensitivities to these mitochondrial localizing drugs appears to be due to the lack of functional mitochondria as a target, and the lowered $\Delta\Psi_{\text{mt}}$, in the ρ^0 cell, which leads to reduced accumulation of Rho 123 and Saffranin O in this latter cell type. Since Rho 123 acts as an uncoupler in isolated mitochondria, and it is dependent on the strength of both $\Delta\Psi_{\text{mt}}$, and plasma $\Delta\Psi$ for intracellular accumulation, it is not surprising to find ρ^0 cells to be more resistant than wt cells to this drug. This, however, does not imply that Rho 123, or other mitochondrial localizing drugs kill the cell by inhibiting oxidative phosphorylation. It is not clear from our results whether a normally functioning cell with intact mitochondria will die if their mitochondria become uncoupled.

The fact that ρ^0 cells (cell model (B)) are able to grow without undergoing oxidative phosphorylation demonstrates that blockage of this process alone does not lead to cell death. It can be argued however, that ρ^0 cells have been selected to survive without oxidative phosphorylation capability and

therefore have developed other mechanisms for this purpose. Thus, they are not equivalent to cells with normally functioning mitochondria that are treated with uncouplers (cell model (A)).

5 Nevertheless, the increased accumulation of the positively-charged drugs, Rho 123 and saffranin O, in wt vs ρ^0 cells, due to the increased $\Delta\Psi_{mt}$, in the former cell type, most likely contributes to their increased sensitivities to both drugs. In fact, as mentioned above, Bochet and Godinet,
10 and Barrientos and Moraes, estimate that $\Delta\Psi_{mt}$, of these ρ^0 cells to be 3-4 times lower than wt which agrees with our results that show ρ^0 cells retain 4 times less Rho 123 than wt. Thus, by whatever mechanism Rho 123 ultimately kills a cell, the intracellular level of drug accumulation necessary
15 for this action appears to be facilitated by the strength of the $\Delta\Psi_{mt}$. However, the preliminary data we present with the cell model above, shows that when cells with normally functioning mitochondria, 143B or MCF-7, are co-treated with Rho 123 and glycolytic inhibitors, their survival is
20 compromised. This clearly indicates that mitochondrial agents which inhibit Oxphos can hypersensitize tumor cells to glycolytic inhibitors and that this combinative effect is indeed the mechanism by which they inhibit cell growth and/or kill tumor cells.

25 In contrast, the nuclear localizing chemotherapeutic agent, Dox, is found to be equitoxic in both cell lines of cell model (B), indicating that functional mitochondria are not an important site for its cytotoxic action. Likewise, the tubulin-binding agents, paclitaxel and vinblastine are also
30 found to be equitoxic in these cell lines. Moreover, as mentioned above with cell model (A), these drugs do not hypersensitize tumor cells to glycolytic inhibitors. Therefore, this data also acts as an important negative

control to demonstrate that compounds which are not known to preferentially accumulate in mitochondria or interact with its function, do not hypersensitize tumor cells to glycolytic inhibitors. In fact, this data further indicates that both
5 cell models (A) and (B) are specific in testing whether inhibition of Oxphos leads to hypersensitization to glycolytic inhibitors which results in growth inhibition and or cell death.

It should be noted here that the reason that ρ^0 cells do
10 not display an MDR phenotype to the chemotherapeutic agents we have tested, even though they do not undergo Oxphos, is because they are growing rapidly in vitro. This is quite different from the in vivo situation where a cell in the middle of a tumor due to a number of reasons, are slow-growing
15 and thus are resistant to most chemotherapeutic agents which target rapidly dividing cells. It is the hypoxic environment however that leads to the anaerobic metabolism these inner core tumor cells undergo, which makes them selectively sensitive to glycolytic inhibitors. This is the essence of
20 our discovery.

As predicted, ρ^0 cells are more sensitive to 2-dg than wt cells showing an ID50 of 50 as compared to 1000 mg/ml for wt cells. Thus out of the six drugs presented in **Table 1** above, 2-dg is the only one that ρ^0 cells are hypersensitive to.
25 Preliminarily we have found similar differential sensitivity to oxamate and iridoacetate.

Thus, our data clearly support our working model that inhibition of Oxphos by either mitochondrial agents (cell model (A)) or by mutation in mitochondrial DNA, ρ^0 cells, (cell
30 model (B)) renders both of them hypersensitive to glycolytic inhibitors. The third part of our equation, cell model (C), is at the center of our studies, in that tumor cells growing

under hypoxic conditions are hypersensitive to inhibitors of glycolysis. The present invention, based on the differences and similarities of our three cell models should lead to a new approach to chemotherapy.

5 One of the problems with successful treatment of solid tumors is eradicating all of the tumor cells including those that are more centrally located. Due to their location, cancer cells at the inner core of the tumor are less oxygenated and therefor rely more on anaerobic metabolism in
10 order to survive. In this condition they divide less rapidly than the more aerobic tumor cells and thus are more resistant to the standard chemotherapeutic agents which take advantage of rapidity of replication as a selective mode of toxicity. In addition, anaerobic cells are less able to produce free
15 radicals when treated with chemotherapeutic agents or irradiation which utilize this part of the cell's capability in order to further potentiate its mode of action. Thus, anaerobiosis in these instances act as another component of resistance to successful chemotherapeutic and radiation
20 treatment.

It should be noted that a gradient of anaerobiosis exists within tumors, therefor it is expected that the more anaerobic the cell becomes the more susceptible it becomes to glycolytic inhibitors, such as 2-deoxyglucose, oxamate and iodoacetate
25 (the latter has been shown to block glycolysis at the glyceraldehyde 3-phosphate dehydrogenase step) and the analogs disclosed in this application. On the other hand, the more aerobic the cells become, the more sensitive they are to conventional chemotherapy and or radiation.

30 Therefore, we propose that glycolytic inhibitors, such as 2-deoxyglucose, oxamate, iodoacetate and the analogs we specify here, significantly increase the efficacy of standard cancer chemotherapeutic and radiation regiments as well as new

protocols emerging with anti-angiogenic agents by selectively killing anaerobic tumor cells when given in combination with the above mentioned therapies.

Specifically, we have discovered that 2-dg or glycolytic inhibitors in general can be added to standard chemotherapy protocols that now exist for carcinomas and sarcomas, a few of which are listed below as representative examples, i.e.:

Breast cancer: Cytosan, Adriamycin, and 5FU, or Taxotere;

10 Sarcomas: Adriamycin I.V. + Cisplatin I.A. (intra arterial) or Ifosphamide Adriamycin and DTIC (all I.V.);

Lung cancer: Navelbine and Cisplatin or Taxotere + Carboplatin or Gemcitabine + Cisplatin;

Small Cell Lung cancer: VP-16 + Cisplatin;

15 Head and Neck cancer: 5FU and Cisplatin;

Colon cancer: 5FU + Leukovorin + CPT-11 (Irinotecan);

and

Multidrug resistant (MDR) tumors: MDR tumors will be treated with a glycolytic inhibitor and drugs active against
20 MDR tumors. Example: combination of 2-dg with Annamycin. Such combinations will be especially useful against breast cancer.

We also disclose treatment which combines blockers of oxphos with glycolytic inhibitors for general antitumor
25 therapy. Treatment with the former agents will convert aerobic tumor cells to anaerobic, thus making them hypersensitive to glycolytic inhibitors. Blockage of Oxphos can be accomplished with agents that, in general, are positively-charged and are relatively lipophilic, examples of
30 which are rhodamine 123, saffranin O, octylpyridium and others. In the case when tumor uptake is reduced by the presence of drug transporting proteins, such as P-gp or MRP (efflux proteins), this treatment will be used in combination

with agents that block efflux, e.g. verapamil, cyclosporin.

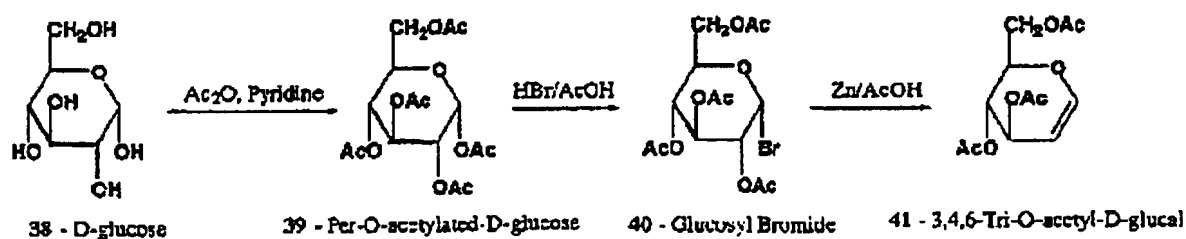
Alternatively, lipophilic cationic compounds which inhibit oxidative phosphorylation in intact cells, but are not recognized by MDR transporting systems, such as
 5 alkylguanidinium analogs, can be used in the MDR + aerobic tumor cells to convert them to anaerobic cells and thereby render them hypersensitive to glycolytic inhibitors.

Moreover, glycolytic inhibitors are effective as single agent treatments for tumors which are mostly anaerobic and
 10 because of their size and location cause discomfort and blockage of normal function, i.e. head and neck tumors. These inhibitors are useful in reducing tumor size when administered orally, IV, IP or directly into the tumor.

Another application of the glycolytic inhibitors of the
 15 present invention is in the treatment of bacterial infections which involve anaerobes, such as clostridia, bacteriodes, etc.

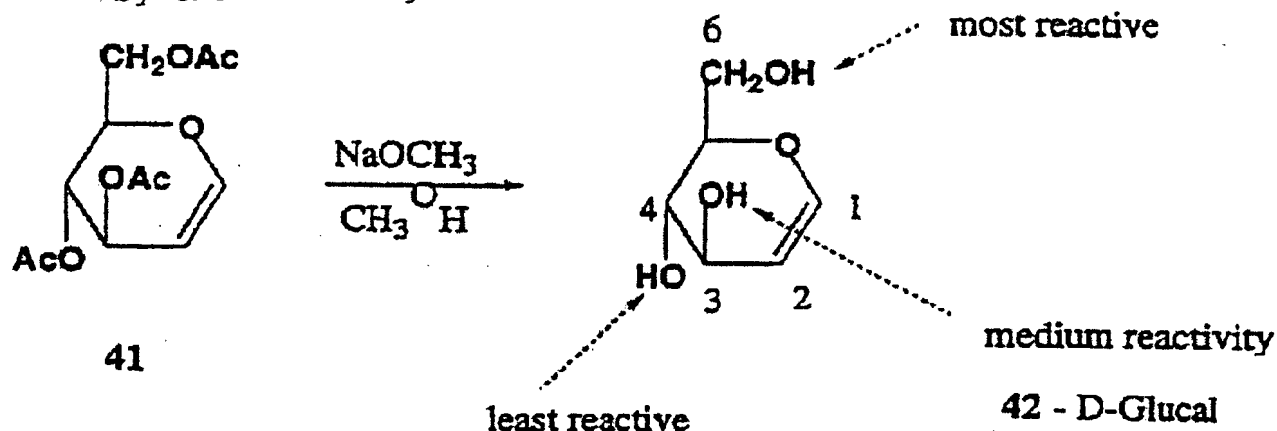
Detail Syntheses of Novel Compounds:

Synthesis of the novel compounds can be achieved by those of ordinary skill in the art by understanding the following
 20 examples. Synthesis of 3,4,6-Tri-O-acetyl-D-glucal can be performed by the scheme:

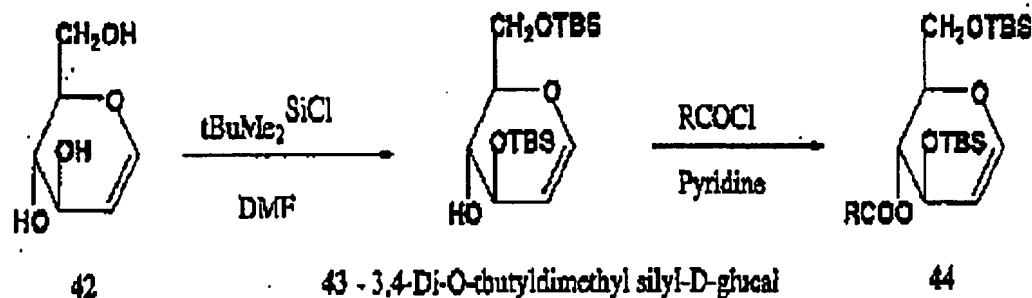


Compound 41 is then deacetylated to glucal 42, which is a key intermediate for the invention. First, it is deoxygenated at C-2 as 2-deoxy-glucose, but more importantly it contains hydroxyl groups that differ in their reactivity. This allows differentiation during blocking reactions and selectivity preparation of acylated derivative of D-glucal. Specifically, D-glucal 42 contains a primary hydroxyl at C-6 which is the most reactive in the molecule. Subsequently the secondary hydroxyl at C-3, because of its allylic position, is more reactive than the secondary hydroxyl at C-4.

Syntheses of D-glucal and its reactivity are illustrated by the following scheme:



4-O-acylated derivative of 2-dg are prepared first. This is done by first blocking hydroxyls at C-6 and C-3, which will leave hydroxyl at C-4 open for acylation. The blocking groups at C-3 and C-6 should be selectively removed in the presence of esters. Such properties are demonstrated by silyl ethers, which are stable in basic but unstable in acidic conditions, whereas acyl groups appear to be stable in the latter conditions. Therefore, D-glucal 42 will first be silylated at C-3 and C-6 with sterically bulky t-butyldimethylchlorosilane (TBS) to 43 leaving a free hydroxyl at C-4 ready for the acylation with acyl chloride to 44 in the following scheme:



TBS = tBuMe_2Si

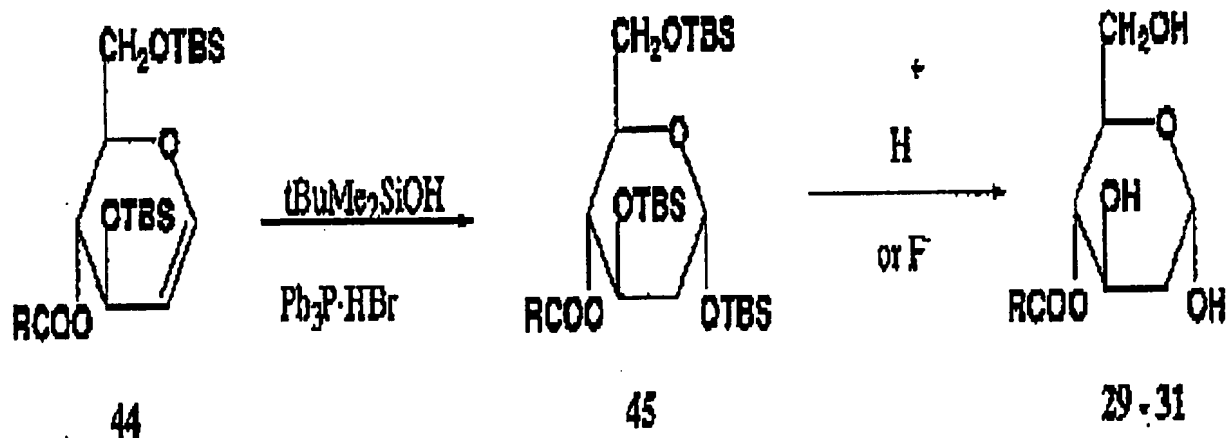
R is an acyl group as shown below

$\text{R} = \text{CO}(\text{CH}_2)_3\text{CH}_3$

$\text{R} = \text{CO}(\text{CH}_2)_{12}\text{CH}_3$

or $\text{R} = \text{CO}(\text{CH}_2)_7\text{CH}_3$

Compound 44 has a reactive double bond, which is reacted with silanol to substitute 2-deoxy-D-glucose 45 in the following scheme. This electrophilic addition reaction requires the presence of triphenylphosphine hydrobromide. 5 Compound 45 is then deprotected selectively at C-1, C-3 and C-6, yielding the desired 4-O-acylated analogs 29-31.



Using similar known strategies the other compound of the invention can be prepared by those skilled in the art without undue experimentation, once the motivations to make these 10 compound, specifically the desire to make glycolytic

inhibitors for the purpose of killing anaerobic cells, have been learned from the present disclosure.

The Novel Method:

Another aspect of the invention is a method of inhibiting
5 one or more steps of the glycolic pathway using an effective
amount of one of more glycolytic inhibitors or schemes, in the
presence of anaerobic cells, specifically for the purpose of
thereby killing the cells. The disclosed compounds, used
singly or in combination, have been shown to achieve this end.
10 Other inhibitors and other inhibiting schemes may also be
used, once the basic premises of the present invention are
understood.

For example, know or new genetic manipulations to inhibit
or block one or more steps in the glycolic pathway may be used
15 to practice the method of the invention.

For a better understanding of the pathway, and in
particular the ten steps of the glycolytic pathway, any one or
more of which may inspire the use of inhibitors or schemes to
thwart for practicing the present invention, the pathway is
20 set forth as follows:

Reactions of glycolysis

<i>Step</i>	<i>Reaction</i>
1	Glucose + ATP \longrightarrow glucose 6-phosphate + ADP + H ⁺
2	Glucose 6-phosphate \rightleftharpoons fructose 6-phosphate
3	Fructose 6-phosphate + ATP \longrightarrow fructose 1,6-bisphosphate + ADP + H ⁺
4	Fructose 1,6-bisphosphate \rightleftharpoons dihydroxyacetone phosphate + glyceraldehyde 3-phosphate
5	Dihydroxyacetone phosphate \rightleftharpoons glyceraldehyde 3-phosphate
6	Glyceraldehyde 3-phosphate + P _i + NAD ⁺ \rightleftharpoons 1,3-bisphosphoglycerate + NADH + H ⁺
7	1,3-Bisphosphoglycerate + ADP \rightleftharpoons 3-phosphoglycerate + ATP
8	3-Phosphoglycerate \rightleftharpoons 2-phosphoglycerate
9	2-Phosphoglycerate \rightleftharpoons phosphoenolpyruvate + H ₂ O
10	Phosphoenolpyruvate + ADP + H ⁺ \longrightarrow pyruvate + ATP

<i>Step</i>	<i>Enzyme</i>	<i>Type</i>	ΔG°	ΔG
1	Hexokinase	a	-4.0	-8.0
2	Phosphoglucose isomerase	c	+0.4	-0.6
3	Phosphofructokinase	a	-3.4	-5.3
4	Aldolase	e	+5.7	-0.3
5	Triose phosphate isomerase	c	+1.8	+0.6
6	Glyceraldehyde 3-phosphate dehydrogenase	f	+1.5	-0.4
7	Phosphoglycerate kinase	a	-4.5	+0.3
8	Phosphoglycerate mutase	b	+1.1	+0.2
9	Enolase	d	+0.4	-0.8
10	Pyruvate kinase	a	-7.5	-4.0

A Rapid Screening Method for Identifying Inhibitors of Oxidative Phosphorylation :

Lactate (or more commonly known as lactic acid) is an end product of glycolysis that is activated when a cell cannot use oxygen as its final electron acceptor. This occurs when cells are metabolizing anaerobically. Thus, under these conditions, pyruvate, which is the last end product of glycolysis during aerobic metabolism, is further metabolized to lactate. We demonstrated that agents such as rhodamine 123 which inhibit oxidative phosphorylation in intact tumor cells (converting

them from aerobic to anaerobic cells) hypersensitize them to inhibitors of glycolysis. This discovery created a need for a screening method to identify agents which inhibit oxidative phosphorylation and thereby render a cell hypersensitive to glycolytic inhibitors. Consequently, we have developed a rapid and an inexpensive way to detect such agents by measuring increases in the production of lactate in living cells.

We have found in our cell models A and B that measurement of the level of lactate correlates with the degree of anaerobiosis. It can be seen in the table below that in cell model B, po cells, which by nature of their deficiency in mitochondrial DNA cannot perform oxidative phosphorylation and are therefore metabolizing anaerobically, produce approximately 3 times more lactate (15.4 $\mu\text{g}/\mu\text{g}$ protein) than their aerobic parental cell counterparts, 143b, (5.4 $\mu\text{g}/\mu\text{g}$ protein). Similarly, in cell model A, (See table below) when the aerobically metabolizing cell, 143b, is treated with increasing doses of rhodamine 123, which should convert it to increasingly anaerobic growth, lactate is accordingly increased as a function of increasing rhodamine 123 doses. In contrast, chemotherapeutic drugs which affect aerobic cells such as Doxorubicin (Dox), Vinblastine (Vbl) and Taxol and do not hypersensitize cells to glycolytic inhibitors, show little or no significant changes in lactate production. We thus provide convincing evidence that increases in lactate is reflective of increases in anaerobic metabolism which can be induced by agents which inhibit mitochondrial oxidative phosphorylation. Such methodology provides a practical means for testing and or screening extensive libraries of structurally diverse compounds to identify inhibitors of oxidative phosphorylation as well as for obtaining new leads to design novel inhibitors of this fundamental cellular

process.

Table 2: LACTATE PRODUCTION IN CELL MODELS A & B

	CELL LINE	DRUG	DOSE ($\mu\text{g/ml}$)	LACTATE ($\mu\text{g}/\mu\text{g}$ protein)
	po	Control	0	15.4 +/- 0.3
5	143b	Control	0	5.4 +/- 0.1
	143b	Rho 123	0.25	9.3 +/- 0.4
	143b	Rho 123	0.50	13.2 +/- 0.2
	143b	Rho 123	1.0	14.6 +/- 2.3
	143b	Dox	0.005	5.1 +/- 0.4
10	143b	Dox	0.01	5.5 +/- 0.7
	143b	Vbl	0.0005	5.6 +/- 0.6
	143b	Vbl	0.001	6.3 +/- 0.9
	143b	Vbl	0.002	5.8 +/- 0.3
	143b	Taxol	0.0005	5.6 +/- 0.7
15	143b	Taxol	0.0010	6.1 +/- 0.7
	143b	Taxol	0.0020	6.6 +/- 0.3

We have thus also demonstrated as part of this invention, that agents such as rhodamine 123 which inhibit oxidative phosphorylation in intact tumor cells, hypersensitize them to inhibitors of glycolysis by accelerating anaerobic metabolism.

Weight Loss Method:

Based on the principle of this discovery, we claim the extension of the use of this concept as a novel approach for weight control as follows:

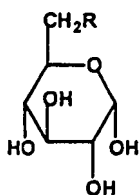
Since anaerobic metabolizing cells take up and use glucose more rapidly than aerobic metabolizing cells, we propose to add small amounts of agents which localize in mitochondria and slow down oxidative phosphorylation. Thus,

most cells in the body would have increased uptake and utilization of glucose, thereby lowering blood sugar levels. Under these conditions, in order to compensate for reduced glucose levels in the blood, gluconeogenesis would be increased by the liver. Gluconeogenesis is a process whereby glucose is synthesized by breakdown of fats. Thus, our treatment effectively increases the breakdown and utilization of stored fat. Currently, intense energetic exercise accomplishes the same thing most likely by a similar mechanism.

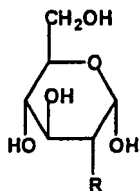
While specific embodiments of the invention have been shown and described in detail to illustrate the application of the principles of the invention, it will be understood that the invention may be embodied otherwise without departing from such principles.

WHAT IS CLAIMED IS:

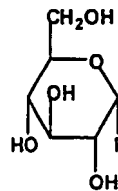
1. A composition comprising at least one of the compounds 1 to 37:



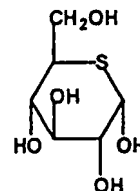
1 - R = F (6-Fluoro-D-glucose)
2 - R = SH (6-Thio-D-glucose)



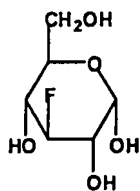
3 - R = H - 2-Deoxy-D-glucose (2-dg)
4 - R = Br - 2-Bromo-D-glucose
5 - R = F - 2-Fluoro-D-glucose
6 - R = I - 2-Iodo-D-glucose



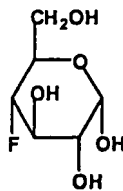
7 - Glucosyl fluoride



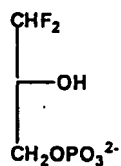
8 - 5-Thio-D-glucose



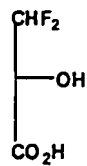
9 - 3-Fluoro-D-glucose



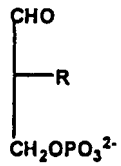
10 - 4-Fluoro-D-glucose



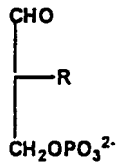
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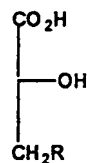
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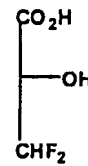
13 - R = I
14 - R = F
15 - R = SH
16 - R = OMe



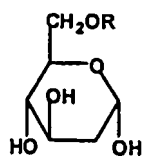
17 - R = I
18 - R = F
19 - R = SH
20 - R = OMe



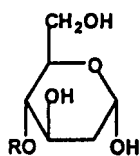
21 - R = I
22 - R = F
23 - R = SH
24 - R = CO₂H



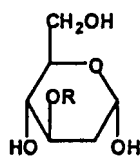
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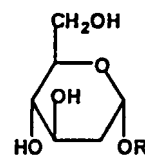
6-O-Esters of 2-dg
26 - R = CO(CH₂)₃CH₃
27 - R = CO(CH₂)₁₂CH₃
28 - R = CO(CH₂)₁₄CH₃



4-O-Esters of 2-dg
29 - R = CO(CH₂)₃CH₃
30 - R = CO(CH₂)₁₂CH₃
31 - R = CO(CH₂)₁₄CH₃



3-O-Esters of 2-dg
32 - R = CO(CH₂)₃CH₃
33 - R = CO(CH₂)₁₂CH₃
34 - R = CO(CH₂)₁₄CH₃



1-O-Esters of 2-dg
35 - R = CO(CH₂)₃CH₃
36 - R = CO(CH₂)₁₂CH₃
37 - R = CO(CH₂)₁₄CH₃

2. A method of killing anaerobic cells comprising
5 administering an effective amount of a composition containing
at least one of the compound 1 to 37 of claim 1.

3. A method of killing cells that are capable of using
steps of the glycolytic pathway to create ATP for cell energy,
comprising inhibiting at least one step of the glycolytic
10 pathway by administering an effective amount of at least one
glycolytic inhibitor.

4. A method according to claim 3, including first
treating aerobically growing tumor cells with an effective
amount of a composition which inhibits oxidative
15 phosphorylation to convert the aerobically growing tumor cells
into anaerobically growing tumor cells which are thus made
hypersensitized to at least one glycolytic inhibitor, and
thereafter administering the effective amount of the at least
one glycolytic inhibitor.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/07173

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/18, 31/67, 31/185, 31/661, 31/7012

US CL : 514/23, 25, 95, 120, 129, 432, 578

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/23, 25, 95, 120, 129, 432, 578

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST, CAPLUS, MEDLINE

search terms: glycolysis, oxidative phosphorylation, deoxyglucose

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,420,489 A (WHISTLER) 13 December 1983, see entire reference	1-3
X — Y	LAMPIDUS, T. J. et al "Selective toxicity of rhodamine 123 in carcinoma cells in vitro" Cancer Res. February 1983, vol 43, pages 716-720, see especially abstract and page 718.	3, 4 — 1, 2
X	US 4,840,939 A (LEVEEN et al) 20 June 1989, see entire reference.	3

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

09 MAY 2001

Date of mailing of the international search report

01 JUN 2001

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INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US01/07173

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	MIKO, M. et al "Effect of chloro- and bromo-derivatives of isocrotonic acid on bioenergetic processes in Ehrlich ascites cells and isolated mitochondria" Neoplasma 1979, vol 26, pages 449-460. See entire reference.	3 ----- 4
X ----- Y	HALICKA, H. D. et al "2-Deoxy-D-glucose enhances sensitivity of human histiocytic lymphoma U937 cells to apoptosis induced by tumor necrosis factor" Cancer Res. January 1995, vol 55, pages 444-449. See abstract and page 448.	3 ----- 4
A	MAZUREK, S. et al "The role of phosphometabolites in cell proliferation, energy metabolism, and tumor therapy" J. Bioener. Biomem. 1997, vol 29, pages 315-330. See entire article.	1-4